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Immunopotentiating Activity of the Water-soluble Lignin Rich Fraction Prepared from LEM—The Extract of the Solid Culture Medium of *Lentinus edodes* Mycelia—

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The water-soluble lignin in LEM (the extract of the solid culture medium of *Lentinus edodes* mycelia) has been known to have antiviral and immunopotentiating activities *in vivo* and *in vitro*. The water-soluble lignin rich fraction (JLS-18) was prepared from LEM using ultrafiltration and hydrophobic column chromatography. JLS-18 showed about 70 times higher antiviral activity than LEM *in vitro*. JLS-18 activated the cytotoxicity of NK cells and macrophages, and activated T cells *in vitro*. JLS-18 also induced interleukin 6 (IL-6) secretion from human leukocytes infected with Sendai virus *in vitro*. These data showed that JLS-18, the water-soluble rich fraction of LEM, had antiviral and immunopotentiating activities.

Key words: JLS-18; water-soluble lignin; LEM; immunopotentiating activity

LEM (the extract of culture medium of *Lentinus edodes* mycelia) has biological activities such as antiviral and immunopotentiating effects. LEM neutralizes the infectivity of the tobacco mosaic virus,[1] and inhibits the cytopathic effect of herpes simplex virus (type 1 and type 2), western equine encephalitis (WEE) virus, poliovirus, measles virus, and mumps virus.[2,3] LEM also inhibits the infection and cytopathic effects of human immunodeficiency virus (HIV), including inhibition of the enzymatic activity of the reverse transcriptase of HIV.[4,5] In the clinic, it has been reported that orally administered LEM is effective on AIDS,[6,7] and chronic type B hepatitis patients.[8,9] In addition, LEM can activate the murine macrophage functions,[10–13] and promote the proliferation of bone marrow cells *in vitro*.[11] Such activities are thought to be derived partially from the water-soluble lignin of LEM.[2,10,11]

In this study, we prepared the water-soluble lignin rich fraction (JLS-18) from LEM and evaluated the antiviral and immunopotentiating activity of it. For the preparation of JLS-18, ultrafiltration and hydrophobic column chromatography were used. The antiviral activity was assessed based on the inhibition of the infectivity of human herpes simplex virus type I (HSV-I) to the mammalian cells *in vitro*. To investigate the effects of JLS-18 on T cells, NK cells, and macrophages, the screening protocols for biological response modifiers established in the National Cancer Institute were used.[14] We also examined the effects of JLS-18 on interleukin 6 (IL-6) secretion from human peripheral leukocytes *in vitro*.

Materials and Methods

Materials. All the chemicals not specified were purchased from Wako Junyaku Co., Ltd. Established cell lines and fetal bovine serum (FBS) were purchased from Dainippon Pharmaceuticals Co., Ltd. Culture media were purchased from Nissui Pharmaceuticals Co., Ltd. Animals were purchased from Charles River Japan Inc.

Preparation of JLS-18. LEM was prepared as previously reported.[1] Briefly *Lentinus edodes* mycelia were cultured in a solid medium composed mainly of sugar-cane bagasse for 3 months, then the whole medium containing mycelia was extracted by hot water and aseptically filtrated.

LEM was separated into three fractions by ultrafiltration (Millipore Co.). The fraction with molecular mass between 3×10^4 and 1×10^6 daltons was pooled and further purified using hydrophobic column chromatography (Octyl-Cellulofine, Biochemicals Co.) as followed. To the fraction, an equal volume of 0.5 M phosphate buffer (pH 7.5) containing 2 M ammonium sulfate was added and it was applied to the column equilibrated with binding buffer (0.25 M phosphate buffer containing 1 M ammonium phosphate). The column was washed with binding buffer and eluted with 0.1 M phosphate buffer containing 0.4 M ammonium sulfate, and then with water. The latter fraction was desalted and stored at 4°C after lyophilization (named JLS-18).

Assay for antiviral activity. Samples were dissolved with assay medium (RPMI-1640 medium with 10% FBS) and distributed in microtiter plates (50 μl/well). NC-37 cells (human lymphoblast) were suspended with the medium and added to each well (1×10^4 cells in 50 μl/well). Then herpes simplex virus type 1 (F strain) was added to each well (2.5×10^3 pfu in 50 μl/well). After 1 hour of incubation at 37°C, the medium was changed and the cells were cultured further for 3 days. The cells were stained with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) and the amount of viable cells was measured from the absorbance at 546 nm. 1 unit of antiviral activity was defined as the amount of the sample which gave 50% inhibition of viral infection.

Analysis of lignin. The amount of lignin was measured by the acetyl bromide method.[15] 2 mg of sample was dissolved with 2.5 ml of 25% (w/w) acetyl bromide in acetic acid and 0.1 ml of 70% perchlolic acid in a glass tube. After incubation at 70°C for 30 min, the reactant was transferred to a plastic tube containing 10 ml of 2 M NaOH and 12.5 ml of glacial acetic acid. After adjusting the volume to 50 ml with acetic acid, the absorbance was measured at 280 nm. Reagent lignin (Nacalai Tesque Co.) was used as a standard.

Analysis of sugar. Samples were dissolved with water at a concentration of 10 mg/ml, and 225 μl of sample solution was mixed with 50 μl of trifluoroacetic acid and incubated at 100°C for 3 hours under 100% nitrogen to hydrolyze sugar to monosaccharides. After lyophilization, the samples were dissolved with water and analyzed by chromatography (Aminex HPX-87N column, Bio Rad). The amount of sugar was measured

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Abbreviations: HIV, human immunodeficiency virus; HSV-I, herpes simplex virus type I; IL-6, interleukin 6; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; EMEM, Eagle's minimum essential medium; LPS, lipopolysaccharide; HBSS, Hank's balanced salts solution; EHAA, extra high amino acids.

as the sum of the amount of each monosaccharide. A mixture of reagent glucose, galactose, xylose, mannose, and arabinose was used as the standard.

Analysis of amino acid composition. 1 mg of sample was dissolved with 6N HCl containing 1% phenol and heated at 150°C for 1 hour under 100% nitrogen. Then the amino acids were analyzed using Beckman 6300 amino acid analyzer. The amount of protein was measured as the sum of the amount of each amino acids.

NK cell cytotoxicity assay. Spleen cells containing NK cells (effector cells) of C3H mouse (male, 3 weeks old) were suspended with RPMI-1640 medium with 10% FBS. The samples dissolved with the medium were added to the cell suspension. To the control culture, only the medium was added. After incubation for 24 hours, the cells were collected by centrifugation and suspended with the new medium. WEHI-164 cells (target cells, mouse fibrosarcoma) labelled with [^{3}H]proline (Amersham) were distributed to each well of a microtiter plate (1×10^4 cells in 100 μl/well). Then the spleen cells incubated with the samples were added to the same plate (12.5×10^4 cells in 100 μl/well) and mixed with the target cells. After incubation for 16 hours, the cells were collected and washed twice with HBSS (Gibco), and the radioactivity remaining in the target cells was counted by a beta counter. PolyI:C (Sigma) was used as the positive control.

Macrophage cytotoxicity assay. Peritoneal macrophages of an ICR mouse (male, 7 weeks old) were suspended with Eagle's minimum essential medium (EMEM) with 5% FBS and distributed to each well of a microtiter plate (1.25×10^5 cells in 100 μl/well). To this plate, samples dissolved with the medium at appropriate concentrations were added (100 μl/well). After incubation for 24 hours, the culture supernatant was removed and L1210 cells (target cells, mouse lymphoblastic leukemia) labelled with [^{125}I]IUdR (Amersham) were added to the plate (1×10^4 cells/200 μl) and mixed with macrophages stimulated with the samples. The cells were incubated further for 16 hours and the radioactivity of the culture supernatant liberated from the target cells was counted by a gamma counter. Lipopolysaccharide (LPS, Calbiochem) was used as positive control.

Allogenic mixed lymphocyte reaction assay. Spleen cells were collected from two different strains of mice, C3H and C57 BL/6 (male, 6 weeks old), and suspended with HBSS. One part of spleen cells of C3H and C57 BL/6 were incubated with 10 μg/ml of mitomycin C (Sigma) at 37°C for 30 minutes, washed with Hank's Balanced Salts solution (HBSS) three times, then suspended with EHAA medium with 0.5% normal mouse serum (stimulator cells). Untreated spleen cells from C3H were used as responder cells. Samples were dissolved with the medium and distributed to a microtiter plate (50 μl/well). To the plate, stimulator cells and responder cells were added (7.5×10^4 cells in 100 μl/well and 5×10^5 cells in 50 μl/well respectively) and incubated for 3 days. Then 1 μCi of [^{3}H]thymidine (Amersham) was added to each well and incubated further for 20 hours. Then the cells were collected and the radio activity incorporated in the responder cells was counted using a beta counter. Thymocin (Becham Co.) was used as the positive control.

IL-6 Production assay. Human peripheral leukocytes were prepared as described previously.[16] The cells were suspended with HAM-F12 medium with 10% FBS (2×10^7 cells/ml). Sendai virus (U.S. Bio Product, NY) was added to the cell suspension at a concentration of 100 hemagglutinin units/1×10^7 cells. The cell suspension was distributed to a 24-well culture plate (1 ml/well) and mixed with same volume of the medium containing samples, and incubated for 18 hours. The concentration of IL-6 of culture supernatant in each well was measured using human IL-6 ELISA kit (Amersham).

Results and Discussion

The antiviral activities and characteristics of JLS-18 and LEM

In each step of preparation of JLS-18 from LEM, the antiviral activity of each fraction was measured, and the fraction that showed the higher antiviral activity was further purified. Both JLS-18 and LEM inhibited the virus infection, but JLS-18 showed about 70 times higher antiviral activity than LEM (Table I). That is, JLS-18 and LEM inhibited the infection by 50% at the concentration of 0.57 μg/ml and 38.3 μg/ml respectively. The recovery of antiviral activity was 18.3% in the process of preparation of JLS-18 from LEM. From 4000 liters of LEM (dried weight was 31,040 g), 84.3 g of JLS-18 could be obtained.

Table I. Comparison of Antiviral Activities of LEM and JLS-18

	Total amount (g)	Specific activities (units/mg)	Total activities (units $\times 10^{-3}$)	Yield (%)
LEM	31,040.0	26.1	808,933	—
JLS-18	84.3	1,752.0	147,690	18.3

Table II. Chemical Composition of LEM and JLS-18

	Lignin	Sugar	Protein	Ash	Other	(%)
LEM	27.4	33.9	10.4	13.5	14.8	
JLS-18	76.4	21.3	2.3	N.D.	—	

The chemical composition of LEM and JLS-18 is shown in Table II. The ratio of lignin of LEM was 27.4%, but it increased up to 76.4% in JLS-18. These date showed that JLS-18 was the water-soluble lignin rich fraction of LEM. Lignin is a macromolecular substance and insoluble in water, but the lignin in LEM was thought to be degraded partially by the lignase of the mushroom, and as a result become water-soluble.[17] The characteristics of water-soluble lignin of LEM have been reported previously.[18,19] In these reports, it was suggested that water-soluble lignin of LEM had polyanionic characteristics. The main component of LEM was sugars which shared 33.9%, but the ratio decreased to 21.3% in JLS-18. The composition of sugar was 70% of xylose, 20% of arabinose, and 8% of glucose (data not shown). The protein shared 10% of LEM, but only 3% of JLS-18. JLS-18 did not contain ash which shared 13.5% of LEM.

Comparing the composition of JLS-18 and LEM, only the water-soluble lignin increased its ratio in JLS-18. In addition, it was reported that the antiviral activity was closely related to the lignin content in the process of preparation of JLS-18 previously.[18] Therefore it was suggested that high antiviral activity of JLS-18 should be attributed to the water-soluble lignin. A similar observation had been reported on LEM.[2,13] The polyanionic macromolecules including dextran sulfate,[20] polyacrylic acid, and polymethacrylic acid,[21,22] have been known to reduce the viral infections. Polyanionic characteristics of the water soluble lignin might contribute to the inhibition of virus infection. There were other reports that the polysaccharide contributed to the antiviral activity of LEM.[4] But the ratio of sugar decreased from 60% to 23% through the purification process of JLS-18 and glycosidase treatment had no effect on antiviral activity of JLS-18 (data not shown). Protease treatment also had no effect, so we concluded that the antiviral effect of JLS-18 was derived from water-soluble lignin.

The in vitro effect of JLS-18 on macrophages, NK cells and T cells, and IL-6 secretion from peripheral leukocytes

To examine the effect of JLS-18 on T cells, NK cells, and

macrophages, we followed the screening protocols for biological response modifiers established by the National Cancer Institute with slight modifications.[15] These protocols have been used to evaluate the immunological effects of new substances which might be used as anti-cancer agents.

The effect of JLS-18 on NK cells was studied by the cytotoxicity against fibrosarcoma cells, which are thought to be targeted selectively by NK cells. As shown in Fig. 1, JLS-18 activated NK cells in a dose-dependent manner at the range from 3.3 to 100 μg/ml. JLS-18 showed the percent cytotoxicity comparable to 5 μg/ml of polyI:C used as the positive control at the concentration of 20 μg/ml. These data showed that JLS-18 was an effective activator of T cells. The effect of JLS-18 on macrophage was studied by the cytotoxicity against L1210 cells (lymphoblastic leukemia). As shown in Fig. 2, the cytotoxicity was increased by the stimulation with JLS-18 by dose dependent manner at the range from 0.4 to 11.1 μg/ml, but the effect was almost saturated at the concentration of 3.6 μg/ml. The effect of JLS-18 was less than LPS used as positive control at the concentration 5 μg/ml. The effect of JLS-18 on T cells was investigated by the allogenic mixed lymphocyte reaction, and was indicated as stimulation index (S.I.). As shown in Fig. 3, the S.I. increased at the concentration of JLS-18 from 0.032 to 0.8 μg/ml in a dose-dependent manner. JLS-18 activated T cells most effectively at concentrations of 0.8 μg/ml, but had no effect at the concentration higher than 20 μg/ml. JLS-18 might be toxic to T cells at such the high concentrations. In addition, the effect of 0.16 μg/ml of JLS-18 was higher than that of 25 μg/ml of thymosin used as the positive control. This result showed that JLS-18 was a potent activator of T cells.

Further, the effect of JLS-18 on IL-6 secretion from peripheral leukocytes containing T cells and macrophages was investigated. IL-6 is a cytokine that is secreted from several types of cells including T cells and macrophages, and induces the final maturation of B cells into antibody-forming cells,[23] the maturation of T cells,[24] and stimulates the production of acute phase protein.[25] As shown in Fig. 4, the amount of IL-6 secretion from virus-infected cells increased at the concentration of JLS-18 from 1.37 μg/ml to 333 μg/ml. At a concentration of 111 μg/ml, the amount of secreted IL-6 was almost two times higher than the culture without JLS-18. These data showed that JLS-18 induced the IL-6 secretion from peripheral leukocytes infected with Sendai virus. Similarly JLS-18 induced IL-6 secretion from the virus-uninfected cells. In addition, it was suggested that the activation of T cells with JLS-18 might be caused by the secreted IL-6.

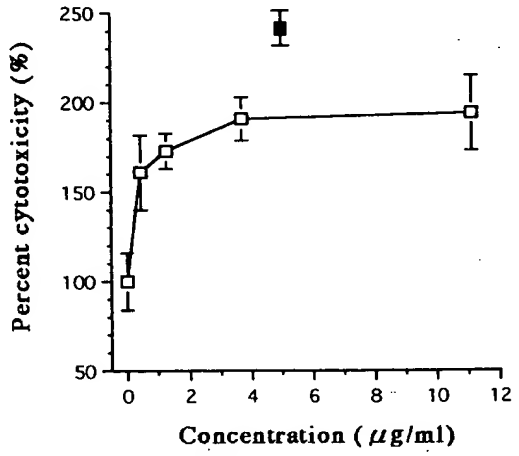

Fig. 2. Macrophage Cytotoxicity Assay.

Peritoneal macrophages were stimulated with various concentrations of JLS-18 (□) or 5 μg/ml of LPS (■) for 24 hours, incubated with L1210 cells (target cells) labeled with [^{125}I]IUdR for 16 hours, then the radioactivity liberated from the target cells was counted. The percent cytotoxicity was calculated by following equation. The values and error bars are the mean ± SD of three independent experiments.

$$\text{Percent cytotoxicity (\%)} = \frac{\text{cpm liberated in the experimental well}}{\text{cpm liberated in the control well}} \times 100\ (\%)$$

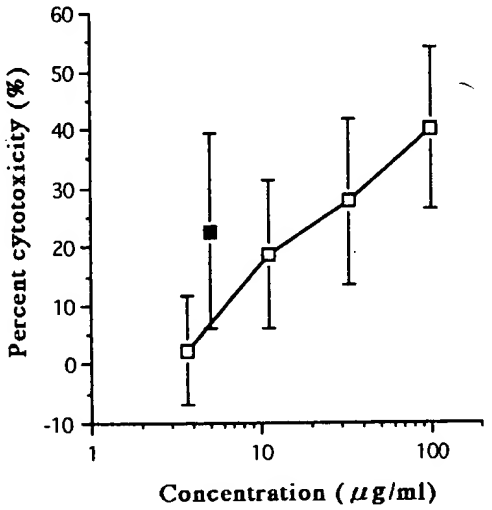

Fig. 1. NK Cell Cytotoxicity Assay.

WEHI-164 cells (target cells) labelled with [^{3}H]proline were cultured with NK cells in spleen cells stimulated with various concentrations of JLS-18 (□) or 5 μg/ml of polyI:C (■). Then the radioactivity remaining in each well was counted. Cytotoxicity of NK cells were indicated as percent cytotoxicity which was calculated by the following equation. The values and error bars are the mean ± SD of four independent experiments.

$$\text{Percent cytotoxicity (\%)} = \left(1 - \frac{\text{cpm in the experimental well}}{\text{cpm in the control well}}\right) \times 100\ (\%)$$

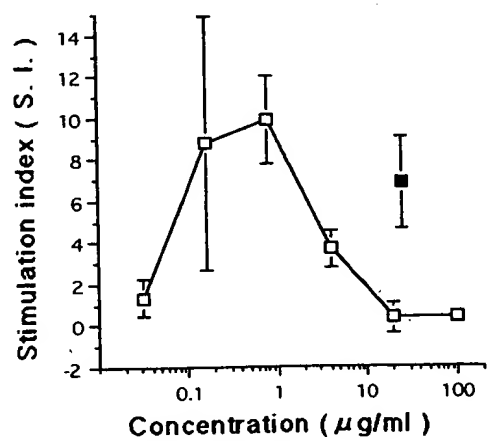

Fig. 3. Allogenic Mixed Lymphocyte Reaction Assay.

Spleen cells containing T cells from C3H and C57 BL/6 mice were treated with mitomycin C and used as stimulator cells for allogenic and control culture respectively. Live spleen cells from C3H (responder cells) were mixed with stimulator cells at the ratio of 1:12.5 and incubated for three days in the medium containing various concentrations of JLS-18 (□) or 25 μg/ml of thymosin (■). Then the cells were labeled with [^{3}H] for 16 hours and the thymidine uptake by T cells was counted. T cell activation was indicated as stimulation index (S.I.) which were calculated by the following equation. The values and error bars are the mean ± SD of three independent experiments.

$$\text{S.I.} = \frac{\text{cpm in the allogenic culture well} - \text{cpm in the control culture well}}{\text{cpm in the control culture well}}$$

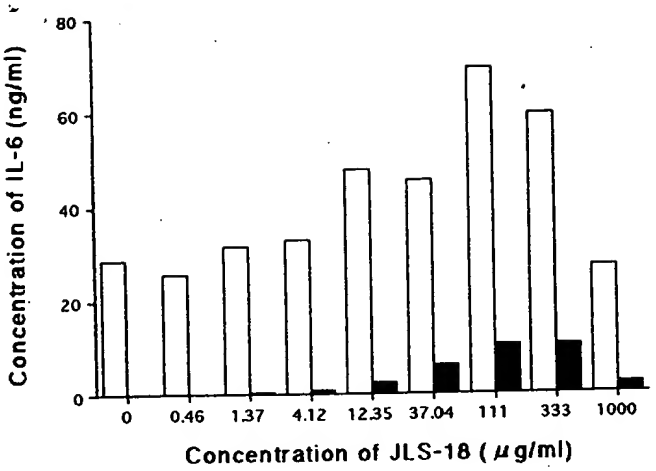

Fig. 4. IL-6 Production Assay.

Human peripheral leukocytes infected with Sendai virus (open bar) or not (solid bar), were stimulated with various concentrations of JLS-18 for 18 hours. Then IL-6 secreted from the cells was measured with a human IL-6 ELISA kit (Amersham). The values are the mean of three independent experiments.

There are clinical reports that the condition of patients with chronic B hepatitis and AIDS has been improved by the oral administration of LEM.[6-9] It has been suggested that improvement of the condition of these patients might have occurred through the immunopotentiating activity of LEM.[8,9] Our *in vitro* study showed that JLS-18 could activate macrophages, NK cells, and T cells and induce IL-6 secretion from leukocytes infected with virus or not. JLS-18 might be effective on the patients with chronic B hepatitis and AIDS through such immunopotentiating activities and useful for the therapy of these diseases.

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